# Isolation, Culture, and Transplantation of Rat Hepatocytic Precursor (Stem-like) Cells

## (43663)

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*Abstract.* **From a review of past studies and the report of new studies from our laboratory, this article provides strong evidence to show that WB-F344 (WB) rat liver epithelial cells are stem-like precursor cells for hepatocytes. WB cells are structurally and phenotypically simple epithelial cells that were isolated from the liver of an adult male Fischer 344 rat, under conditions that excluded their origin from hepatocytes *in vivo*. WB cells express a phenotypic repertory that overlaps, but is distinct from, that of both hepatocytes and bile duct epithelial cells. The complex phenotype of WB cells is compatible with their being embryonic or undifferentiated variants of either hepatocytes or bile duct epithelial cells. When WB cells are tagged genetically with genes for bacterial β-galactosidase and neomycin resistance (BAG2-WB), they and their progeny can be distinguished from parental WB cells and hepatocytes by the expression of these gene products. Progeny of BAG2-WB cells that were transplanted into the liver parenchyma of syngeneic rats integrated into hepatic plates and acquired the morphological and functional attributes of adjacent host hepatocytes; the progeny of BAG2-WB cells in the liver express albumin, tyrosine aminotransferase, α-1-antitrypsin, and transferrin. We also demonstrate that progeny of BAG2-WB cells can be recovered from livers into which they have been transplanted, which may allow the elucidation of alterations in gene expression that accompany their differentiation.** [P.S.E.B.M. 1993, Vol 204]

In this article, we present the results of our recent studies on the transplantation into the livers of syngeneic animals and the retrieval from these livers of WB-F344 rat liver epithelial cells that have been engineered to contain transgenes for *Escherichia coli* β-galactosidase and Tn5 neomycin resistance. In conjunction with our past studies on the isolation, culture, and characterization of propagable rat liver epithelial cell lines, which we summarize here, our recent results provide strong support for the presence of a population of epithelial stem cells for hepatocytes in the normal liver of the adult rat.

The existence of an epithelial stem cell in livers of adult animals is controversial (for recent reviews, see 1–4). The debate surrounding the existence of the putative liver epithelial stem cell is fueled by the fact that the concepts of stem cells and their functional properties are largely intuitive; stem cells, including those that may exist in the liver, cannot be identified microscopically in tissues or in cellular suspensions isolated from tissues (5). Even after the partial purification of bone marrow stem cells using antibodies to surface antigens, their presence can be inferred only by their ability to re-establish lineages of differentiated bone marrow cells when they are transplanted into an animal from which the marrow has been ablated (6). The requirement for a population of stem cells to serve as the source of cellular renewal in tissues that turn over rapidly, such as bone marrow, intestinal epithelium, and epidermis, is compelling. However, this is not the case for liver epithelial stem cells. The long life-span (low turnover) of hepatocytes in the adult liver (for a review, see 7) and the fact that differentiated hepatocytes retain the capacity for self-renewal in response to cell loss (8, 9) mitigate against the need for a liver epithelial stem cell. Recent claims that the kinetics and pattern of cell proliferation in the normal livers of adult rats indicate the existence of a generative compartment fed by stem

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cells (10–12) ignore a large volume of contradictory data from previous studies on the cell population dynamics of the liver (7, 8).

Evidence supporting the existence of liver epithelial stem cells comes mainly from three sources: the founding of epithelial lineages from hepatoblasts during embryogenesis of the liver (13–15); the re-establishment of epithelial lineages in adult animals from poorly differentiated cells (so-called oval cells) that have proliferated pathologically (16–18); and the isolation from livers of adult animals and culture *in vitro* of clonogenic cells that can differentiate into hepatocytes and/or bile duct epithelial cells when they are transplanted into appropriate sites *in vivo* (19, 20). It is not surprising that cells possessing some stem-like properties can be isolated and cultured from embryonic livers of fetal rats in which lineage development is taking place (21), and from pathologic livers of adult rats in which new epithelial lineages are being established from proliferated oval cells (22–24). In contrast, our studies have concentrated on the evaluation of the liver of the normal adult rat for the presence of liver epithelial cells with stem-like properties. Several years ago, we were intrigued by the concept of a conditional or facultative epithelial stem cell in livers of adult animals, and we focused our attention on the nonhepatocytic liver epithelial cell as a potential precursor cell for hepatocytes (25, 26). Some of the phenotypic properties that we found to characterize cultured nonhepatocytic liver epithelial cells, especially high clonogenicity, ready propagability in culture, and simple structural and functional features, clearly distinguished them from the differentiated hepatocytes and bile duct epithelial cells, and seemed to be most compatible with properties expected of stem cells (5).

## Culture of Liver Epithelial Cells

Our early studies on explant cultures demonstrated the distinctive morphologic and histochemical characteristics of hepatocytes, biliary duct epithelium, and nonhepatocytic epithelial cells, which we called clear epithelial cells, that grow out as monolayers of cells from cultured liver explants (27). Subsequent development of techniques to prepare viable suspensions of dispersed liver cells, by perfusing the liver with a solution of collagenase (28), has enabled the separate isolation of each of these (and other) types of cells directly from the liver and their establishment in culture (for review, see 29). In the course of our studies, we have established in culture numerous lines of liver epithelial cells and determined their phenotypic properties. Most of the results of the past studies we review and the results of new studies we describe here have come from the analysis of one specific line of cultured liver epithelial cells, which we have designated WB-F344 or WB cells (30).

## Phenotypic Characteristics of WB-F344 Liver Epithelial Cells

The WB-F344 rat liver epithelial cell line was isolated in our laboratory from a normal adult rat by primary cloning, a condition that excluded mature hepatocytes as the cells of origin (30). WB cells are small (9–15 $\mu$m diameter) polygonal cells that grow in a monolayer (Fig. 1A). Adjacent cells are joined by numerous desmosomes (30), nexus junctions contain connexins 26 and 43 (31), and adjacent cells are dye coupled (32). Cells are polarized, surfaces directed to the media interface contain microvillus-like projections, and a basement membrane-like material accumulates on the substrate surface (30). WB cells have a quasidiploid karyotype that has remained stable during subculture (30). They do not proliferate in soft agar and are nontumorigenic (30). Major phenotypic properties of WB-F344 cells are listed in Table I. WB cells share some phenotypic properties with both hepatocytes and bile duct epithelial cells, but their overall phenotype differs distinctively from either differentiated cell type (30). In general, based on their phenotypic properties, WB cells could be considered immature variants of either hepatocytes or bile duct epithelial cells. A highly simplified phenotype might be expected to characterize an undifferentiated stem cell; alternatively, this phenotype may reflect merely the adaptation to conditions of culture *in vitro*.

## Studies on the Origin of Cultured Liver Epithelial Cells

Liver epithelial cells with the general phenotypic characteristics of WB cells are readily isolated from normal livers of rats of any age and established in culture. Despite the ease with which they may be recovered, the origin in the normal liver tissue of these functionally simple epithelial cells is uncertain. The controversy over the origin *in vivo* of cultured liver epithelial cells began even in the era of explant cultures. The major possibilities for the derivation of cultured simple epithelial cells were posited to be hepatocytes or bile duct epithelial cells, poorly differentiated epithelial cells that might exist in some obscure tissue niche, and liver endothelium or mesothelium (27, 40). The subsequent availability of molecular markers of cell type (particularly antibodies to cytokeratins) appears to have excluded the possibility that liver epithelial cells originate from hepatic endothelial cells, but the question of their potential origin from hepatic mesothelium remains unresolved (41, 42). Primarily, this uncertainty reflects the fact that epithelial cell lines cultured from liver cells (including WB) weakly express (or do not express) cytokeratins and other intermediate filaments, and those that are expressed tend to overlap with the patterns of expression of these proteins by mesothelium of Glisson's capsule (41). Studies with monoclonal an-

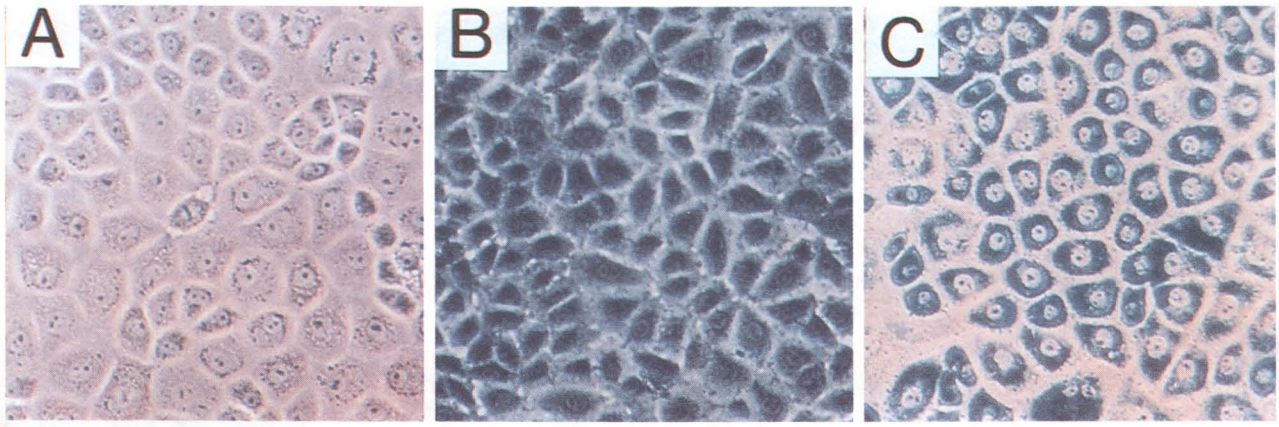


**Figure 1.** Morphology of WB-F344 rat liver epithelial cells and progeny in monolayer culture. (A) WB-F344 cells viewed by phase contrast microscopy; (B) and (C), BAG2-WB cells and BAG2-WB-H3 cells (respectively) demonstrating intense blue reaction product from $\beta$-galactosidase histochemical reaction (magnification ×275).

**Table I.** Major Phenotypic Properties of WB-F344 Rat Liver Epithelial Cells

| |
|---|
| Express numerous high affinity receptors for epidermal growth factor (33) |
| Respond to EGF, transforming growth factor-$\alpha$ (33–35) and transforming growth factor-$\beta$ (36) |
| Secrete insulin-type growth factor-II (unpublished observations) and TGF-$\beta$ (37) |
| Do not express glucose 6-phosphatase and do not store glycogen (30) |
| Weakly express $\gamma$-glutamyltranspeptidase biochemically (38) |
| Express hexokinase isozyme type I; lactate dehydrogenase isozymes 3, 4, and 5; aldolase isozymes A and $A_3C$; pyruvate kinase isozyme K; and liver type alkaline phosphatase (30) |
| Express glutathione *S*-transferase P (39) |
| Weakly express albumin and $\alpha$-fetoprotein (30) |
| Weakly express oval cell antigens recognized by monoclonal antibodies Ep. 1 and OV6, and hepatocyte antigen for monoclonal antibody H4 (unpublished observations) |
| Weakly express (or do not express) cytokeratins 7, 8, 18, and 19 (unpublished observations) |

tibodies developed against WB cells show that these cells contain antigens that share epitopes with cells in small ducts of the liver and pancreas, as well as with cells in intestinal crypts and in the tracheobronchial epithelium (A. K. Wennerberg and J. W. Grisham, unpublished observations). All of these different cells share a common embryological derivation from the primitive gut. We have not found any evidence for the reaction of anti-WB cell monoclonal antibodies with mesothelial cells.

Our early work attempted to determine whether hepatocytes were the obligate or possible origin of propagable liver epithelial cell lines in culture. A major hypothesis of the origin of liver epithelial cell lines holds that they are derived from hepatocytes that have lost differentiated properties in culture (3). Since high density cultures of collagenase-dispersed liver cells contain a complex mixture of cell types, it is impossible in mass cultures to trace the origination of the propagable cells from any of the particular types of cells, including hepatocytes, in suspensions of liver cells. We approached this problem from two directions: by establishment of cultured liver epithelial cells from collagenase suspensions in which hepatocytes had been eliminated, and by primary cloning of liver epithelial cells from collagenase suspensions (25, 26). Studies by other investigators demonstrated that hepatocytes are extremely sensitive to the lytic effects of trypsin or Pronase (43). Hepatocytes cannot be identified morphologically in collagenase-dispersed cell suspensions that have been exposed appropriately to either trypsin or Pronase, although nonparenchymal cells remain viable (25, 26). We were able to isolate nearly as many colonies of small epithelial cells from liver cell suspensions depleted of hepatocytes by treatment with strong proteases as from untreated suspensions that contained hepatocytes (Table II).

Primary cloning of cells directly from a cellular suspension was used by Coon (44) to establish cultures

**Table II.** Effect of the Selective Protease-Mediated Destruction of Hepatocytes on the Establishment of Epithelial Cell Colonies from Normal Rat Liver[a]

| Protease employed | Duration of protease treatment (min) | Viable Hepatocytes (%) | No. of epithelial colonies |
|---|---|---|---|
| Trypsin | 30 | 94 ± 11 | 328 ± 128 |
| | 60 | 67 ± 20 | 402 ± 204 |
| | 120 | 9 ± 30 | 298 ± 106 |
| Pronase | 60 | 0 | 503 ± 206 |
| | 120 | 0 | 198 ± 182 |

[a] Data are from Grisham (25).

of functional parenchymal cells from cellular suspensions of tissues. Indeed, Coon established the first well-characterized propagable line of liver epithelial cells (the BRL line) by this method (45), but he apparently did not attempt to identify the type of cell in the suspension that gave rise to clonal lines. In our studies, cells from collagenase suspensions were plated at low densities and the position of individual morphologically identified cells, which were widely separated from adjacent cells, were marked and monitored for their ability to form colonies (25, 26). As shown in Table III, identifiable hepatocytes never proliferated to form epithelial colonies under the culture conditions employed, whereas a small epithelial cell present in the collagenase suspension was efficiently clonogenic, and the colonies could be expanded to yield propagable lines of liver epithelial cells. The results of these studies showed conclusively that hepatocytes in collagenase-dispersed cell suspensions were not the obligate precursors of liver epithelial cell lines. Our studies showed that liver epithelial colonies could arise from small nondescript epithelial cells, which were morphologically distinct from hepatocytes. This conclusion was subsequently confirmed by studies which demonstrated that such small nondescript epithelial cells in collagenase suspensions of liver could be separated efficiently from hepatocytes by differential sedimentation and established separately in culture (46). Our studies also indicated that the small epithelial cells composed a minority of epithelial cells in the liver (less than 1% of liver cells) and that they had a very high primary cloning efficiency (about 2.5%) in culture (25, 26).

The intrahepatic location of these small epithelial cells is still uncertain. The small cells can be isolated from collagenase-dispersed liver suspensions prepared so that portal tracts are not digested and their contents are excluded from the cell suspension (46). This result suggests that the small epithelial cells may reside in the lobular parenchymal compartment. On the other hand, our preliminary studies with monoclonal antibodies to antigens of WB cells have not shown any epitopes to be shared with cells in the liver outside portal tracts, and within portal tracts only in small bile ductules. Thus, the location and function of these cells in the liver remain obscure. Although these results show that liver epithelial cell lines do not require hepatocytes for their origination, they do not exclude the possibility that hepatocytes may give rise to liver epithelial lines under some circumstances.

### Transplantation of Cultured Liver Epithelial Cells

Validation of the hypothesis that the culturable liver epithelial cells function in the liver as conditional or facultative stem cells for hepatocytes or for biliary epithelial cells requires the demonstration that they can be induced by appropriate conditions to acquire the differentiated properties of one or both of these cell types. Most of our studies have been directed toward the analysis of WB cells that have been transplanted into various sites in syngeneic Fischer 344 rats. We first undertook such transplantation studies in cooperation with Jirtle and Michalopoulos, who had successfully transplanted isolated hepatocytes into the interscapular fat pads of rats (47). In these previously unpublished studies, we demonstrated a few small aggregates of cells morphologically resembling hepatocytes in fat pads into which WB cells had been transplanted several weeks earlier (Fig. 2). Because of the small sizes of the aggregates of hepatocyte-like cells and the technical difficulties in analyzing the phenotypic properties of small aggregates of cells in fat pads, we were not able to show that the cells in the fat pads had acquired the hepatocyte phenotype in any way other than morphological resemblance. Nonetheless, these results suggested that transplanted WB cells might acquire characteristics of hepatocytes *in vivo*. Subsequently, we attempted to transplant WB cells into spleens of syngeneic animals, a site that had been shown previously to be suitable for the growth of transplanted hepatocytes (48). Despite numerous attempts, we were never able to identify the cells we had transplanted into spleens or, later, directly into livers. We gained unexpected insights into the differentiation potential of neoplastically transformed WB cells from studies of the tumors produced in syngeneic animals by transplantation of cells that were cloned from a population of WB cells neoplastically transformed by exposure *in vitro* to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These tumors included well-differentiated hepatocellular carcinomas, adenocarcinomas of biliary or intestinal type, and hepatoblastomas (49). These results showed that, at least when transformed,

**Table III.** Clonogenic Properties of Parenchymal and Nonparenchymal Cells from Normal Rat Livers[a]

| Cell type | No. of single cells plated | Epithelial colonies formed | Cloning efficiency (%) |
|---|---|---|---|
| Hepatocytes | 960 | 0 | 0 |
| Nonparenchymal cells | 168 | 4 | 2.4 |

[a] Data are from Grisham (25).

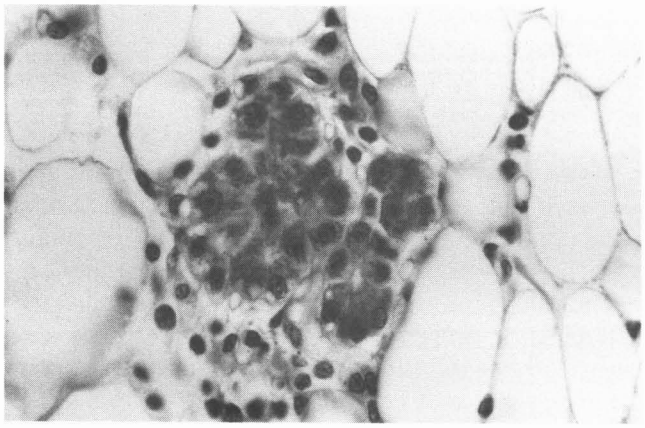

**Figure 2.** Morphology of WB-F344 rat liver epithelial cells after transplantation into the interscapular fat pads of syngeneic rats. Fat pads were collected 3–4 weeks after transplantation of WB cells and paraffin sections were prepared. Cells morphologically resembling hepatocytes were visualized by hematoxylin and eosin staining (magnification ×625).

WB cells are pluripotent for the major differentiated hepatic epithelial phenotypes.

While our previous studies provided intriguing hints that WB cells may differentiate into hepatocytes when transplanted into syngeneic animals, the lack of an effective way to identify the progeny of the transplanted cells prevented the conclusive demonstration that nontumorous WB cells could differentiate into hepatocytes or other hepatic epithelial cells *in vivo*. In order to allow clear identification of progeny of transplanted cells, we have tagged WB cells genetically by infecting them with the packaging defective CRE BAG2 retrovirus (50, 51). Transplanted cells containing the BAG2 construct can be identified by their expression of bacterial β-galactosidase, which is histochemically distinct from the mammalian enzyme. Also, cells tagged genetically with the BAG2 construct carry the gene for neomycin resistance, providing an additional means to identify their progeny and to recover them selectively from cellular suspensions of tissue into which they have been transplanted. This method for marking precursor cells has facilitated the examinations of cell lineage development in tissues *in vivo* (51–53).

## Methods

WB-F344 cells were modified genetically by infection with the packaging defective CRE BAG2 retrovirus (50, 51). The modified cells, termed BAG2-WB (20), express the retrovirally transfected neomycin antibiotic resistance gene from the transposon Tn5 and the *E. coli* β-galactosidase gene (52). The *E. coli* β-galactosidase protein is a histochemically detectable marker for transplanted cells that can be distinguished from mammalian β-galactosidase. BAG2-WB cells that expressed high levels of β-galactosidase activity *in vitro* (Fig. 1B) were utilized for transplantation into adult rats (20). BAG2-WB cells were introduced into the liver tissue of young adult rats (150–200 g) by direct transcapsular injection into the median liver lobe ($5 \times 10^6$ cells injected). Cells intended for transplantation were trypsinized and washed in three changes of ice-cold Thilly's buffered salt solution to remove traces of serum before injection of cells resuspended in Thilly's buffered salt solution (20).

Rats were sacrificed at various times (10–32 weeks) after transplantation of BAG2-WB cells and inspected for the presence of transplanted cells (β-galactosidase-positive cells) within the hepatic parenchyma. Liver tissue was perfused briefly with a solution containing 150 m*M* NaCl, 5 m*M* $MgCl_2$, and 100 m*M* $NaPO_4$ (pH 7.0) to remove blood, followed by *in situ* perfusion with a solution containing 4% paraformaldehyde, 150 m*M* NaCl, and 100 m*M* $NaPO_4$ (pH 7.0) to fix the tissue. The paraformaldehyde-fixed liver tissue was incubated overnight in a solution containing 30% sucrose, 150 m*M* NaCl, 5 m*M* $MgCl_2$, and 100 m*M* $NaPO_4$ (pH 7.0), and then flash frozen in isopentane cooled in liquid nitrogen. Cryosections (8–10 μm) were prepared and postfixed in 1% glutaraldehyde, and β-galactosidase was detected histochemically as described (20).

Immunohistochemistry was performed on liver tissue sections after the histochemical detection of β-galactosidase. Antibodies directed against rat albumin (Cappel/Organon Teknika, Durham, NC), rat transferrin (Cappel/Organon Teknika, Durham, NC), human α-1-antitrypsin (Sigma Immunochemicals, St. Louis, MO) and rat tyrosine aminotransferase (gift of G. Yeoh) were utilized for these studies. Tissue sections were permeabilized by incubation in a blocking buffer containing 10% bovine serum, 0.3% Triton X-100, 150 m*M* NaCl, and 100 m*M* $NaPO_4$ (pH 7.4). Incubations with primary and secondary antisera were carried out in the same buffer containing 0.1% Triton X-100. Biotinylated secondary antibodies were detected using a horseradish peroxidase-conjugated strepavadin system (Vectastain, Vector Laboratories, Burlingame, CA) and diaminobenzidine/NiCl horseradish peroxidase substrate (Vector Laboratories). Control immunostaining reactions were performed without inclusion of a primary antibody or after using nonspecific mouse IgG and conditioned media from mouse myeloma cells that do not produce a specific IgG.

Transplanted BAG2-WB cells were recovered from the livers of rats 6–20 weeks after transplantation. Liver cell suspensions were prepared using the two-step collagenase perfusion technique of Seglen (54). Typically, livers were perfused with 200 ml of calcium-free buffer followed by perfusion with 75–100 ml of 0.5 mg/ml collagenase solution (Worthington Biochemical Corp., Freehold, NJ). Digested liver tissue was minced and subjected to an additional incubation in collagenase for 15 min. Dispersed liver cells were collected and cell

debris and blood cells removed by differential centrifugation. Cell mixtures (containing hepatocytes, nonparenchymal epithelial cells, macrophages, and fibroblasts) were plated onto uncoated plastic dishes and maintained in Richter's improved minimal essential medium with zinc option supplemented with insulin and fetal bovine serum, as described (55). Cells were refed fresh medium 24 h after plating and every 2 days thereafter.

Amplification of the coding sequence of the neomycin resistance gene by polymerase chain reaction was utilized to determine the presence of BAG2-retroviral DNA within genomic DNA samples isolated from the cultured cells recovered from livers. Synthetic oligodeoxynucleotide primers complementary to DNA sequences located in the coding region of the neomycin resistance gene (56) and the SV40 polyadenylation signal (57) of the BAG2 retroviral DNA were used (sense primer: 5′-GATCAAGAGACAGGAGGATCGGTTTCGC; antisense primer: 5′-GGATCCAGACATGATAAGATACATTGATGAG). Thirty-five cycles of amplification with the *Thermus aquaticus* (Taq) polymerase were performed using 95°C for denaturation, 58°C for primer annealing, and 72°C for elongation. In positive samples, the amplification reaction produced a DNA fragment approximately 2 kb in size. Reactions containing WB-F344 DNA as the control template for PCR failed to produce an amplified DNA product.

All studies involving animals used humane methods stipulated in NIH guidelines. Proposals for animal studies were reviewed and approved by the Institutional Review Board of the University of North Carolina at Chapel Hill.

## Results

**Differentiation of BAG2-WB Cells in the Liver.** In livers of rats sacrificed at various intervals after transplantation of BAG2-WB cells, β-galactosidase-positive cells were located in hepatic plates among the host hepatocytes (20). The β-galactosidase-positive cells in hepatic plates were comparable to adjacent host hepatocytes in size, and they contained large, spherical nuclei with prominent nucleoli typical of hepatocytes (Fig. 3, A and B). β-Galactosidase-positive cells were observed in hepatic plates for as long as recipient animals have been studied, to date up to 32 weeks after transplantation of BAG2-WB cells (20; W. B. Coleman, G. J. Smith, and J. W. Grisham, unpublished observations). These observations show that transplanted WB rat liver epithelial cells can integrate into hepatic plates, as do transplanted hepatocytes (58), and that they undergo morphologic changes that cause them to look like hepatocytes (20).

In recent studies, we have demonstrated that the β-galactosidase-positive hepatocyte-like cells also express functional differentiation typical of hepatocytes.

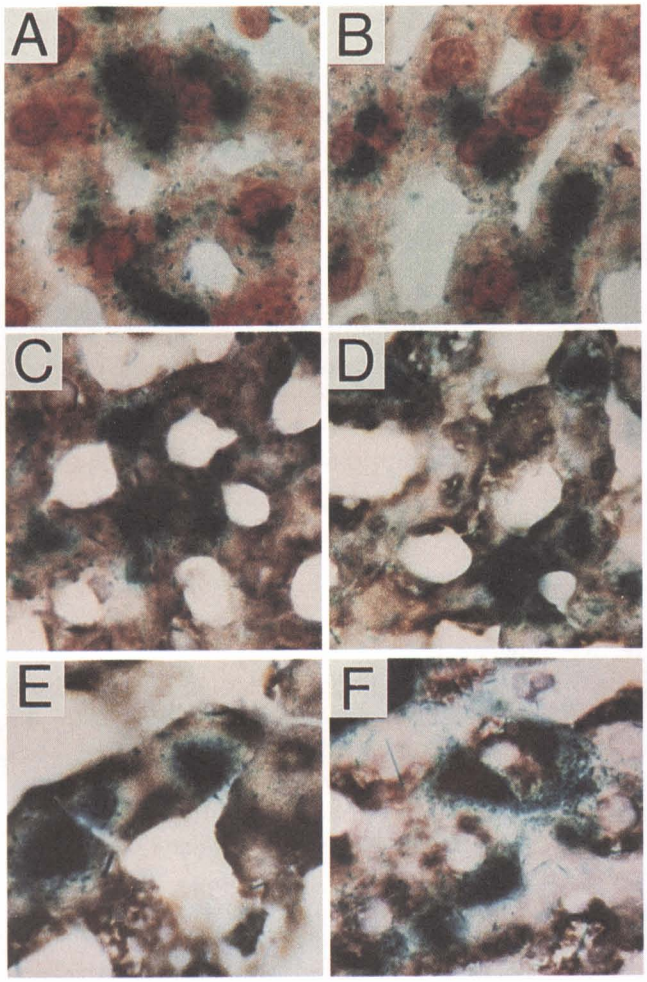


**Figure 3.** Cryosections demonstrating the morphologic and functional differentiation of BAG2-WB cells after transplantation into the livers of syngeneic rats. Liver tissue was obtained at various intervals (10–32 weeks) after transplantation of BAG2-WB cells. Tissue was fixed with paraformaldehyde *in situ* and then prepared for cryosectioning. Cryosections (8–10 μm) were fixed briefly in glutaraldehyde, histochemically stained for β-galactosidase for 12 to 14 hr, and then counterstained with Mayer's hematoxylin (A and B) or immunohistochemically stained for hepatocyte-specific antigens (C–F). The following antibodies were used for immunostaining reactions: goat anti-rat albumin (C); rabbit anti-rat transferrin (D); mouse anti-human α-1-antitrypsin (E); and rabbit anti-rat tyrosine aminotransferase (F) (magnification ×550).

Selected hepatocyte-specific antigens were localized immunohistochemically in tissue sections that had been subjected previously to the histochemical reaction for bacterial β-galactosidase. β-Galactosidase-positive hepatocytes in hepatic plates were also histochemically positive for albumin (Fig. 3C), transferrin (Fig. 3D), α-1-antitrypsin (Fig. 3E), and tyrosine aminotransferase (Fig. 3F). Antibodies to each of these hepatocyte-specific protein products decorated the majority of hepatocytes, both those that were marked by β-galactosidase (progeny of transplanted cells) and those that were not (host hepatocytes). Goat anti-rat albumin antibody and rabbit anti-rat transferrin antibody stained primarily

the cytoplasm of hepatocytes. Rabbit anti-rat tyrosine aminotransferase antibody and mouse anti-human α-1-antitrypsin antibody intensely stained both the cytoplasm and sinusoidal surfaces of hepatocytes. Control immunostaining reactions (minus primary antibody or nonspecific IgG) did not result in the deposition of discernible horseradish peroxidase reaction product on liver tissue sections (data not shown). These results show that β-galactosidase-positive hepatocytes derived from BAG2-WB cells express antigens for albumin, transferrin, α-1-antitrypsin, and tyrosine aminotransferase, as do host hepatocytes (Fig. 3, C-F). The presence of both the β-galactosidase reaction product (blue chromagen) and the horseradish peroxidase reaction product (fine black precipitate) in hepatocytes indicates that transplanted BAG2-WB cells differentiate both morphologically and functionally after transplantation into the liver.

**Recovery of Transplanted BAG2-WB Cells from the Liver.** Our recent studies demonstrate that we can recover progeny of BAG2-WB cells from the livers into which they have been transplanted. Under the culture conditions we used, hepatocytes remained viable for only 12–14 days, and then rapidly disappeared from cultures. Nonhepatocytic epithelial cells proliferated under the same culture conditions, forming visible colonies within 8–10 days. Histochemical staining of primary liver cell cultures 2–3 days after plating them from collagenase suspensions shows both β-galactosidase-positive hepatocytes and nonhepatocytic epithelial cells. This observation suggests the possibility that undifferentiated BAG2-WB cells, as well as their progeny that had acquired hepatocytic properties, are present in collagenase suspensions from livers that received BAG2-WB transplants. However, as we noted previously (20), β-galactosidase-positive macrophages may give a weakly positive histochemical signal after prolonged incubation in the β-galactosidase substrate (20). This cross-reaction complicated the identification in livers of β-galactosidase-positive small nonhepatocytic cells, which are similar in size to Kupffer cells. Other types of cells in livers were never positive for β-galactosidase activity. The epithelial cells that proliferated and formed large colonies in these cultures after 18–21 days exhibited a morphology in culture that closely resembled that of authentic WB cells (30). Some of the cell colonies were strongly positive for β-galactosidase (Fig. 1C), suggesting their origin from transplanted BAG2-WB cells. Epithelial cell colonies established from liver cell suspensions prepared from control rats never demonstrated discernible β-galactosidase reaction product (data not shown). After several weeks in culture, the β-galactosidase-positive cell colonies were trypsinized and subcultured, and the subcultured lines continued to express β-galactosidase activity. Partial characterization of one of these lines, termed BAG2-WB-H3, showed that the cells were derived from transplanted BAG2-WB cells. The origin of this cell line was verified by amplification of the DNA sequence from the neomycin resistance gene portion of the CRE BAG2 retroviral DNA (20) in genomic DNA isolated from BAG2-WB-H3 cells (Fig. 4). The amplification of a DNA product approximately 2 kb in size from the BAG2-WB-H3 cell genomic DNA indicated the presence of the retroviral DNA within the genomes of recovered cells; genomic DNA from endogenous liver cells from the host rat liver does not possess this DNA sequence and could not give rise to an amplified product (20).

These results demonstrate that BAG2-WB cells can be recovered successfully from the livers of adult rats several months after their transplantation. However, we have not yet determined to our satisfaction whether the recovered BAG2-WB-H3 cells originated from hepatocytes derived from BAG2-WB cells or from undifferentiated epithelial progeny of BAG2-WB cells that were sequestered in livers. We have not identified transplanted BAG2-WB cells in sites outside the hepatic plates in sections of liver examined at long intervals after transplanting the cells (20). Nevertheless, it is possible that some undifferentiated BAG2-WB cells are sequestered in unidentified intrahepatic locations that protect the transplanted cells from the strong differentiation-inducing stimuli of the lobular parenchyma, and that preserves their high capacity to proliferate in culture. Alternatively, BAG2-WB cells may possess the capacity to reattain rapidly their original proliferative

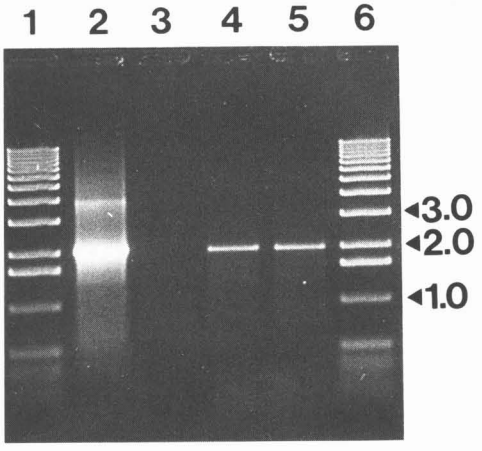


**Figure 4.** Detection by polymerase chain reaction (PCR) of BAG2-retroviral DNA in genomic DNA prepared from cultured epithelial cells. Genomic DNA was prepared from BAG2-WB cells, BAG2-WB-H3 cells, and WB-F344 cells. PCR reactions were analyzed on 0.8% agarose gel containing 40 m*M* Tris-acetate/1 m*M* EDTA (pH 7.6), and stained with ethidium bromide. Molecular size standards (1-kb ladder, Stratagene, La Jolla, CA) are shown in Lanes 1 and 6. PCR amplification products from the following DNA template sources are shown: Lane 2, positive control pSV2neo plasmid DNA; Lane 3, WB-F344 genomic DNA; Lane 4, BAG2-WB cell genomic DNA: and Lane 5, BAG2-WB-H3 genomic DNA.

**Table IV.** Evidence of the Differentiation Potential of WB Cells

| |
|---|
| Morphologically resemble hepatocytes after transplantation into interscapular fat pads of syngeneic rats (this article) |
| Tumors produced by neoplastically transformed WB cells express various differentiated states, including hepatocellular carcinomas, hepatoblastomas, adenocarcinomas, and epidermal carcinomas (49) |
| Incorporate into hepatic plates and morphologically differentiate into hepatocyte-like cells after transplantation into liver of adult rats (20) |
| Functionally differentiate (express albumin, transferrin, $\alpha$-1-antitrypsin and tyrosine aminotransferase) after transplantation into livers of adult rats (this article) |

potential despite their apparent hepatocytic maturation *in vivo*. Future studies will address these possibilities.

## Discussion

Taken together, results of our past and recent studies support the general concept of the presence of a population of stem-like cells that can differentiate into hepatocytes in the livers of adult rats (Table IV). Our studies demonstrate clearly that livers of adult rats contain small, highly clonogenic, nonhepatocytic epithelial cells; after isolation, culture, and transplantation back into the liver parenchyma, these nonhepatocytic epithelial cells integrate into the hepatic plates of host hepatocytes and acquire morphologic and functional properties of differentiated hepatocytes. We conclude, therefore, that cells of the WB-F344 rat liver epithelial cell line, the cultured counterpart of the small *in vivo* epithelial cell, are stem cell-like precursors of hepatocytes. Small, morphologically simple WB cells are induced to differentiate by the microenvironmental influences of the lobular parenchymal compartment of the liver. In the liver, BAG2-WB cells incorporate into hepatic plates where they quadruple in size and acquire the complex phenotype of host hepatocytes. The microenvironment of the lobular parenchyma appears to exert a powerful influence on WB cells to differentiate into hepatocytes, since we have not yet found undifferentiated BAG2-WB cells in sections of liver, nor have we yet found evidence that transplanted cells have differentiated into bile ducts. However, since we have observed progeny of transplanted BAG2-WB cells in livers only in the hepatic plates of the lobular parenchymal compartment, a site unlikely to induce the acquisition of biliary epithelial differentiation by these cells, our studies do not answer the question of whether WB cells can differentiate along bile duct lineages when an appropriate (but unknown) stimulus is provided. Intrahepatic bile ducts form from fetal hepatoblasts under the inductive influence apparently provided by their contact with the mesenchyme of portal tracts (13, 59, 60). It is possible that transplanted BAG2-WB cells may require contact with portal mesenchyme, or the equivalent stimulus, to produce bile ducts. Alternatively, WB cells may not possess the bipotential capability to differentiate into both hepatocytes and biliary duct epithelial cells. We doubt that this hypothesis is correct, since neoplastically transformed WB cells are able to form tumors that express either hepatocytic or biliary ductal differentiation (49). We suspect rather that we have not yet transplanted BAG2-WB cells into the appropriate tissue microenvironment required to induce biliary duct epithelial differentiation.

The ability to recover BAG2-WB cells from livers into which they have been transplanted, as well as to identify the progeny of these cells while they are in the liver, provides a potential means to examine further the intrahepatic source of cultured rat liver epithelial cells, as well as to attempt to pinpoint the location of the precursor cells in the liver. Specifically, it should be possible to determine unequivocally whether differentiated hepatocytes can give rise to propagable lines of liver epithelial cells after undergoing culture-induced loss of differentiated properties, as well as whether some transplanted cells localize in a tissue niche *in vivo* that "protects" them from the stimulus to undergo hepatocytic differentiation. The ability to trace the fate of genetically marked cells may make it possible to evaluate the question of transdifferentiation (modulation) between hepatocytes and bile duct epithelial cells *in vivo* under different pathologic conditions. Furthermore, the ability to recover from livers the progeny of transplanted BAG2-WB cells may enable the elucidation of the microenvironmental conditions that induce hepatocytic differentiation, as well as the qualitative changes in gene expression that accompany and sustain the differentiated state.

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